FILE NAME: ALCOA (ALC)

DATE: 1971 May

DOC#: ALC045

DOCUMENT DESCRIPTION: Journal Article - An Experimental Approach to Study the Toxicity of Nonparticulate Air Pollutants

An Experimental Approach to Study the Toxicity of Nonparticulate Air Pollutants

I. Rationale and Methods

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This report describes an experimental model for studying the toxicity of nonparticulate air pollutants, in terms of a 'biochemical lesion.' Changes in chemical composition of lung tissue homogenate, following inhalation of a noxious gas, could neither be traced to a particular tissue component, nor be regarded as solitary unrelated incidents. Biochemical changes in a single kind of cell, separated from the respiratory tract after the animal's exposure to poisonous gas, may be more directly related to the toxic mechanism of the investigated gas. A technique for isolating epithelium lining the rat trachea has been adapted from a method used to separate tracheal and bronchial epithelia from human autopsy material. Ozone was chosen as test gas in this experimental approach because of its importance in photochemical smog and its presence in certain industrial environments.

THIS IS the first of two reports dealing with the development of an experimental model for the study of the toxicity of nonparticulate air pollutants. The second report deals with the effect of inhalation of ozone on the levels of the coenzyme, nadide phosphate (NADP), in the tracheal mucosa of the rat.

Experimental studies on the toxicity of air pollutants have been largely limited to changes in structure and functions of the lungs and bronchial tree, mucous secretion and ciliary motion, deposition and clearance mechanisms, and chemical changes in lung tissue homogenates.

Research tended to focus on those effects which were easiest to measure, often at the expense of the more delicate and less detectable changes. The investigation of respiratory function, for example, has probably been overemphasized in comparison to other physiological and biochemical parameters.[1]

Specific therapy or prophylaxis for diseases caused by toxic inhalants (and affecting the respiratory system) is lacking, in spite of numerous investigations that have been conducted in this field.

The study of biochemical changes in various tissues has been one means of elucidating disease mechanisms. In reference to air pollutants, some investigators, eg, Scheel et al[2] and Buell et al,[3] have described changes in the chemical composition of lung tissue homogenates following inhalation of ozone. The interpretation of their findings, in terms of the mechanism of toxicity of ozone, presents several difficulties. The lung, unlike

Submitted for publication May 14, 1970; accepted June 1.

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the liver, for example, is composed of a more heterogeneous assortment of cells and tissues that are dissimilar in structure as well as function. Thus, a change in the chemical composition of lung tissue homogenates can neither be traced to a particular tissue component, nor could it be regarded as a solitary incident. It may represent a profile of a multitude of changes, a "vector" of several forces operating simultaneously. Furthermore, unless the chemical changes in the different components of a lung tissue homogenate are additive, they may elude the investigator by nullifying each other.

The approach adopted by Spencer[4-6] in the study of the role of smoking in the etiology of bronchial carcinoma has been adapted to the purpose of this investigation. He attempted to detect precancerous 'biochemical lesions' by the correlation of biochemical changes in tracheal and bronchial epithelia with smoking habits and clinical records. Spencer's approach[4] required the development of a method for the removal of tracheal and bronchial epithelia from human autopsy specimens. In this investigation his technique has been modified for the preparation of epithelial cell suspensions from the trachea of the rat.

This investigation was planned with two goals in mind. The first goal was to develop an experimental model for the study of the toxicity of air pollutants, in terms of 'biochemical pathology.' A second goal was to try to elucidate some of the aspects of the basic mechanism of ozone toxicity. Ozone was chosen as the test gas because of its prevalence under various environmental circumstances, notably its prominence in certain kinds of 'smog.' It also shares with other air pollutants, eg, oxides of nitrogen and organic peroxide compounds, the two properties of being both an oxidant and an irritant. Ozone is an important component of the Los Angeles smog.[7,8] During periods of high photochemical activity, it accounts for 90% or more of the total oxidant, while nitrogen dioxide is the main component when photochemical activity is low or absent.[8] The presence of ozone and its effects on human health in inert-gas-shielded arc welding have been described by several authors.[9,10]

Methods

Isolation of Tracheal Epithelium.—Thirty-six young, adult male rats of the CFN strain were used in this investigation. The animals ranged in age from two to four months, and in weight from 200 to 395 gm. The rats were killed by stunning, and the tracheas were immediately excised through a midline longitudinal incision. A stream of ice-cold isotonic sucrose-0.01M edetic acid (EDTA) solution was squeezed into the trachael lumen from a plastic bottle through a fine-tipped nozzle, in order to remove the mucus and blood aspirated by some animals during death. The esophagus was then excised and the trachea slit open along its dorsal surface. The trachea was then trimmed and further cleaned of mucus in a Petri dish, using a soft camel-hair brush and ice-cold sucrose-edetic acid solution.

The technique used for separation of the epithelial cells lining the trachea was adapted from the one reported by Spencer.[4] A large rubber stopper 4 inches in diameter, covered with filter paper, was used (Fig 1). The open trachea was fixed on the rubber stopper with insect pins, the trough formed facing upwards. Small volumes of ice-cold sucrose-edetic acid solution, 0.01 to 0.02 ml, were instilled on the mucosal surface through disposable capillary-glass micropipets. The epithelium was brought into suspension using a No. 2 camel-hair brush, with bristles trimmed to a length of approximately 4 mm. The sequential steps of instilling sucrose-edetic acid solution, brushing off the epithelium, and pipetting the cell suspension out of the trachea were repeated until a total volume of 0.2 to 0.5 ml had accumulated. The sides, as well as the bottom, of the tracheal trough were brushed in order to harvest the greatest number of cells.

The following procedure was used for cell counting: After repeated passing of the cell suspension in a microsyringe, 0.03 ml of 0.1% methylene blue in the isotonic sucrose-edetic acid solution, pH 7.4, was added to an equal volume of epithelial suspension in the same medium. In order to achieve thorough mixing and further cell dispersion, the contents of the tube were again passed several times through a microsyringe. Counts of nuclei were made in a hemacytometer chamber in the same fashion as in counting white blood cells. The standard error of counting was estimated by the method of Berkson et al.[11]

Exposure of Animals to Ozone.—The ozone exposure apparatus is illustrated in Fig 2. Ozone was generated from pure oxygen (Ultra High Purity Oxygen) obtained from a commercial

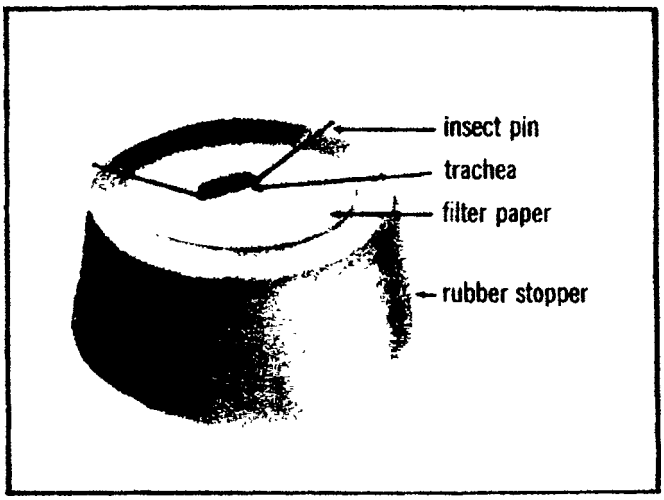


Fig 1.—Apparatus used for isolation of epithelial cells from the trachea of the rat.

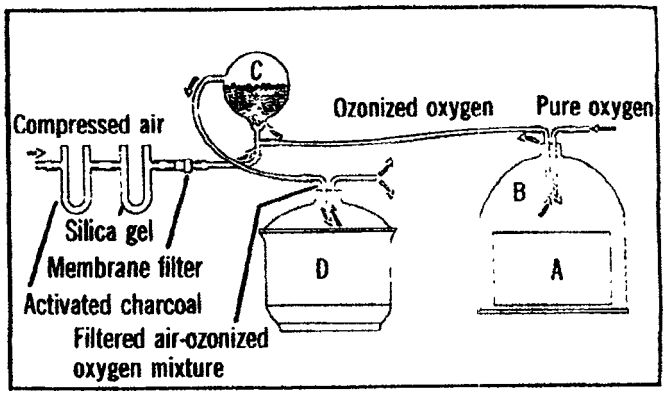


Fig 2.—Diagram of the ozone exposure apparatus, showing ozone generator (A), bell jar (B), mixing vessel with glass beads (C), and animal inhalation chamber (D).

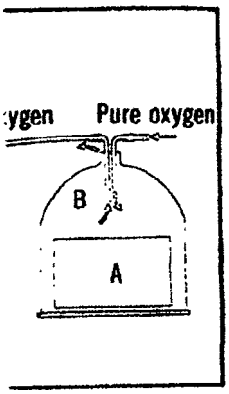
manufacturer. Pure oxygen was preferred to air for the purpose of generating ozone in order to avoid simultaneous generation of nitrogen oxides (from the nitrogen in the air). Svirbely and Saltzman[12] reported that substances associated with ozone production from air (including oxides of nitrogen) are unlikely to influence the toxicity of ozone in laboratory animals. Since this study was planned to investigate some aspects of the basic mechanism of injury by ozone, and as an experimental model for toxicity studies of other inhalants, the above-mentioned precaution was taken.

An ozone generator (Welsone R-2), labeled *A* in Fig 2, was obtained from a commercial manufacturer. It was housed in a glass bell jar (*B* in Fig 2) tightly fitting over a circular glass plate, on which the ozonator was placed. The inflow of pure oxygen to the bell jar was controlled by a two-stage valve regulator connected to the oxygen cylinder and measured with a gas flowmeter.

Ozonized oxygen flowed through flexible tubing from the bell jar to a spherical mixing vessel (*C* in Fig 2). This vessel is 2,000 ml in capacity and was filled with glass beads 6 mm in diameter. Filtered partially-dried air was introduced into the mixing vessel through another inlet. This arrangement enhanced efficient mixing of air and ozonized oxygen. A stopcocked outlet, through which the air-oxygen-ozone mixture flowed to the animal inhalation chamber (*D* in Fig 2), was built in the mixing vessel at a level higher than the uppermost surface of the glass beads. The air used for diluting ozonized oxygen was supplied from a compressed-air line. It was passed through activated charcoal to remove any mineral oil that might have contaminated it from the compressor, and through silica gel to dry it to a relative humidity of 40% to 50%. The air was then filtered through a membrane filter, 0.8μ in pore size.

A glass desiccator, 8,000 ml in capacity, was used as an animal inhalation chamber. A thick sheet of aluminum, with several holes drilled

—Apparatus used for iso-epithelial cells from the f the rat.



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into it, was placed on the protrusions in the inside of the glass desiccator approximately 4 inches above its bottom. This perforated aluminum sheet served as a platform on which the animal was placed during exposure to ozone, and helped to separate the rat from its excreta. The opening in the lid of the desiccator was fitted with a glass-to-glass cover through which entered inlet and outlet tubes equipped with stopcocks. Air flowed from the outlet of the inhalation chamber into a laboratory hood in which the whole exposure apparatus was placed. The use of a glass desiccator as an inhalation chamber was adapted from Diggle and Gage.[13]

Vinyl tubing (Tygon) was used as the tubing material for flexible connections in the entire apparatus. Temperature in the inhalation chamber was measured with an ordinary mercury thermometer. Relative humidity was measured with an indicator manufactured by an industrial instrument company. The relative humidity indicator was calibrated with a dry and wet bulb thermometer. All gas flowmeters were obtained from a commercial scientific company and calibrated with a spirometer (Collin's).

Sixteen animals were used for comparison of the ratio of reduced nadide phosphate (NADPH) to oxidized nadide phosphate ($NADP^+$) in normal animals to that ratio in animals poisoned with ozone. The weight of the control animals (mean ± SE) was 331 ± 20 gm, while that of the experimental group was 337 ± 14 gm. Exposure to ozone was assigned at random. The animals were exposed for 1 hour to 33 ppm ozone in an air-oxygen mixture, supplied to the inhalation chamber at a rate of 1,000 ml air and 50 ml oxygen per minute. Samples for analysis of the ozone concentration were taken at the inlet of the inhalation chamber and analyzed by the neutral buffered-potassium iodide method described by Saltzman.[14]

Results

During the exposure period the animals became lethargic after a few minutes, and soon their breathing was labored. Gross examination of the lungs after inhalation of ozone revealed congestion and subpleural hemorrhages. Figures 3 and 4 illustrate the effect on the lungs of ozone inhalation at a concentration of 33 ppm. Histological examination of the trachea, excised immediately after exposure of the animal to ozone, revealed no abnormality that could be detected with the light microscope.

The extent of removal of the epithelium by this method was studied by examining histological sections of the trachea before and after separation of the cells. Figure 5 illustrates the appearance of the trachea of a normal rat. Figures 6 and 7 show the appearance of the trachea after the epithelium had been removed by the brush method. The submucosal layer remained intact. The absence of contamination of the epithelial cell suspension with connective tissue was verified by phase-contrast microscopic examination of unstained cell smears and examination of stained preparations. The appearance of the cells soon after they were separated is illustrated in Fig 8. The brush technique resulted in removal of sheets and clumps of epithelium. Repeated passing of the cell suspension in a micropipet effected partial separation of the cell clumps. Repeated passing through the orifice of a microsyringe resulted in better separation of the cells. Figure 9 demonstrates the effect of repeated passing of the cell suspension through the orifice of a micropipet, while Fig 10 shows the results of using a microsyringe for the same purpose. Considerable breakage of the cells took place when the microsyringe was used to disperse the clumps. The cells were ground in tissue homogenizer in preparation for the assay of $NADP^+$ and NADPH. Figure 11 illustrates their appearance after homogenization.

The technique developed for the assay of coenzyme made a total cell count neither feasible nor essential. However, it was considered desirable to obtain an estimate of the total number of cells isolated from one trachea. Such an estimate was only approximate, since complete separation of the cell clumps into individual cells was not attained. The number of cells that could be isolated from a trachea was of the order of $1.56 \times 10^6 \pm 2\%$ (mean ± CV). In this study the coefficient of variation is the standard error, rather than the standard deviation, calculated as percentage of the mean. For the purpose of this investigation, we considered this way of expressing the coefficient of variation more appropriate for assessment of reproducibility.

Comment

The relevance of studying the epithelium

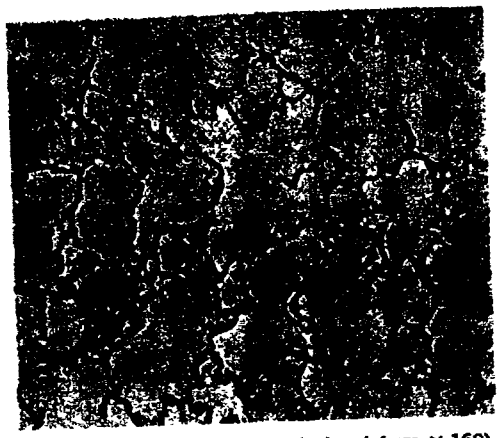


Fig 3.—Lung of a normal rat (reduced from × 160).



Fig 4.—Lung of a rat which inhaled ozone for 1 hour at a concentration of 33 ppm (reduced from × 160).

Fig 5.—Transverse section in the trachea of a normal rat (reduced from × 400).



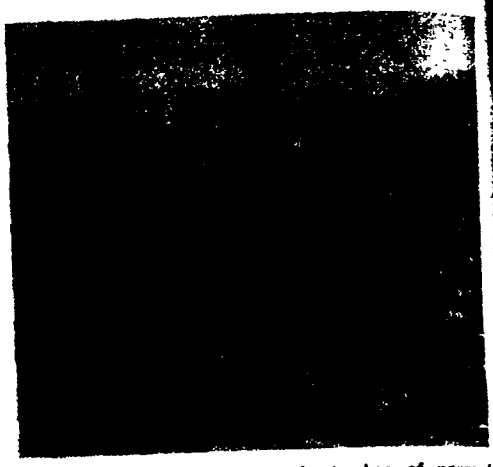


Fig 6.—Transverse section in trachea of normal rat, after removal of the epithelium by brush method. A part of the mucosa, from which ciliated epithelium was not removed, is shown (reduced from × 400).

Fig 7.—Transverse section in trachea of normal rat after removal of the epithelium by brush method. The submucosal layer is intact (slightly reduced from × 400).

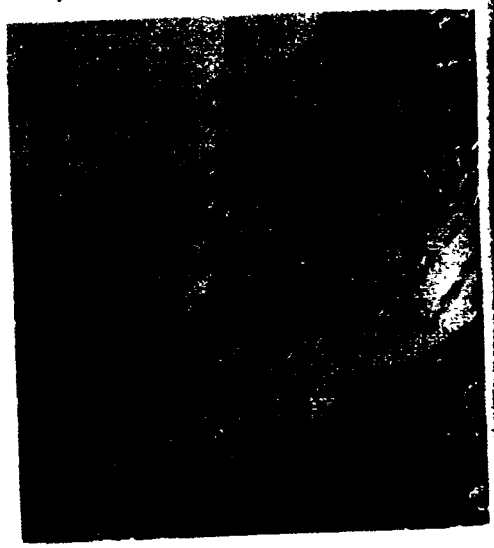




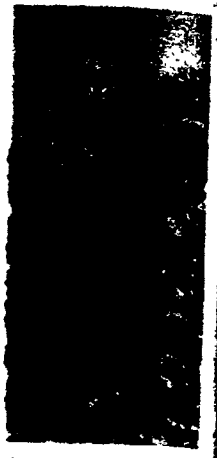
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of the trachea could be questioned, since serious injury to the respiratory tract results from the pulmonary rather than the tracheal lesions. Tracheal epithelium is one of the earlier targets of contact with a highly reactive inhalant. It is biochemically active; it secretes mucus and transports it through ciliary motion. Thus, although tracheal epi-

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Fig 8.—Epithelial cells isolated from the trachea of the rat (slightly reduced from × 640).

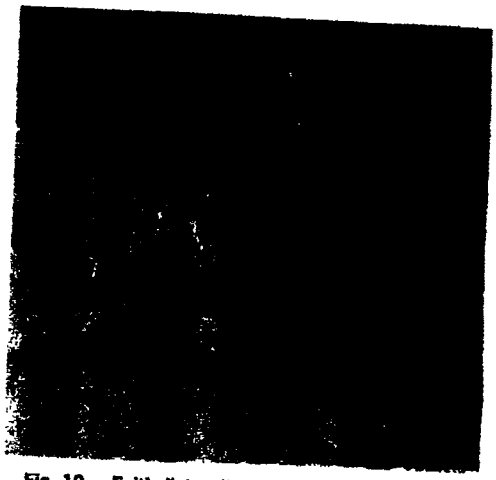


Fig 10.—Epithelial cells isolated from the trachea of the rat. Cell suspension had been passed several times through a microsyringe (reduced from × 400).

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Fig 9.—Epithelial cells isolated from the trachea of the rat. Cell suspension had been passed several times through a micropipet (reduced from × 640).



Fig 11.—Epithelial cells isolated from the trachea of the rat. Cell suspension had been ground in a tissue homogenizer (reduced from × 400).



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helium is not the site of maximal damage when fatal intoxication takes place, it is involved in important mechanisms of defense against toxic inhalants. Also, the epithelium of the trachea can be isolated, relatively free from contamination by other cellular elements. Detection of a 'biochemical lesion' in such a preparation may thus be more meaningful with respect to the basic mechanism of injury caused by the inhalant in question.

This investigation was supported in part by research grant UI-00450 from the National Institutes of Health, by general research grant 5-SO1-FR-05447, from NIH to the School of Public Health, the University of Michigan, and by American Cancer Society institutional grant IN-40H.

The experiments reported in this investigation are part of a thesis submitted by Dr. Nasr to the University of Michigan in partial fulfillment of the requirements for the degree of doctor of philosophy in industrial health.

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